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In vitro propagation of *Campanula glomerata*, 'Acaulis' from leaf blade explants

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Abstract

Development of an in vitro regeneration system for *Campanula* using leaf blades as a source of explants is useful for mass propagation to produce uniform size plants that can be used for evaluation of new hybrids and for investigation of genetic transformation. *Campanula glomerata*, 'Acaulis' is relatively easy to propagate in vitro as compared to other *Campanula* spp., tested. *C. glomerata*, 'Acaulis' plants cultured in vitro on Murashige and Skoog (MS) basal salts medium containing 1.0 mg/l (4.4 μM) N^6-benzyladenine (BA) and 0.01 mg/l (0.05 μM) α-naphthalene acetic acid (NAA) were the source of leaf explants. Maintenance of plants in vitro in low light irradiance (10 μmol m^{-2} s^{-1}) is recommended because high light irradiance (100 μmol m^{-2} s^{-1}) suppressed regeneration. Maintenance of plants at 14 °C in vitro under low light for at least 2 months resulted in optimum regeneration from leaf blades as compared to 18 °C. Explants obtained from leaves of the in vitro grown plants regenerated best (2–3 shoots/explant) on MS medium supplemented with 4 mg/l (17.6 μM) BA and 0.1 mg/l (0.5 μM) NAA. BA was more effective than 2-isopentenyl adenine (2iP). This is the first successful report of the regeneration from leaf blades of *Campanula*.

Keywords: Perennial; Shoot regeneration; Light irradiance; Temperature; Plant growth regulator

1. Introduction

The genus *Campanula* is a diverse group composed of approximately 300 species that are distributed in the temperate regions of the Northern hemisphere (Crook, 1951).

Abbreviations: BA, benzyl aminopurine; 2iP, N^6-(2-isopentenyl)adenosine; NAA, α-naphthalene acetic acid; Zeatin, 6-[4-hydroxy-methyl-but-ethylamino]purine

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Most perennial *Campanula* species such as *C. glomerata* and *C. medium*, are grown as bedding plants, and they require a cold treatment for shoots to form and to flower under a long day photoperiod (Crook, 1951; Lewis and Lynch, 1989). Most perennial *Campanula* species and cultivars are typically propagated by division of the shoots or by rooting of stems to produce a homogenous population. However, these methods of propagation are time consuming and require many stock plants. Although conventional vegetative propagation methods such as division in perennial plants could make propagules available at a low cost, micropropagation will meet the need to supply sufficient propagules for sale in a short period of time.

In vitro propagation of *C. isophylla* and *C. carpatica*, which are generally grown as potted plants from seeds, has been reported from stem pieces containing axillary or apical buds on MS medium supplemented with BA (Brandt, 1992, 1994). Addition of NAA did not increase the number of shoots regenerated (Brandt, 1992). Contamination in culture was a problem when shoots were obtained from greenhouse grown plants. However, many *Campanula* species produce leaves from their crowns and will not form stems until flowering. Therefore, leaf blades or petioles must be used to start in vitro propagation. There is no information on tissue culture of *Campanula* using leaves as a source of explant to our knowledge.

Success of in vitro regeneration depends on various factors, such as the differences in cultivar, the physiological status of mother plants, plant growth regulators, and development stages of the source explants. In *Pelargonium* × *hortorum* (Agarwal and Ranu, 2000), explants obtained from leaves before expanding and from green branches showed better regeneration than those from expanded leaves and from old and woody shoots. Adventitious shoot regeneration using young leaf explants in *Ocimum basilicum* L. (Phippen and Simon, 2000), *Gardenia jasminoides* Ellis (Al-Juboory et al., 1998) was reported. In *Gardenia*, BA was more effective in regenerating shoots than 2iP treated explants. Adventitious shoots were regenerated from in vitro cultured leaves in *Prunus armeniaca* (Perez-Tornero et al., 2000) or in vitro grown seedlings of *Alstroemeria* L. (Lin et al., 1997). Because perennial type *Campanula* plants enter dormancy after flowering, the time when explants were obtained, the preparation of stock plants before taking explants, or the age of leaves from which explants were obtained will affect the regeneration success.

This investigation was initiated to establish an efficient regeneration method for *Campanula* using leaf blade explants of several *Campanula* cultivars that do not form stems before cold treatment. The objectives of this research were to investigate the effects of temperature, light, and plant growth regulators on the regeneration from leaf blade explants of *Campanula glomerata*, 'Acaulis'.

2. Materials and methods

2.1. Plant material and greenhouse growth

Campanula persicifolia L., 'White Bells' and 'Telham Beauty'; *C. latiloba* A. DC., 'Hidcote Amethyst' and *C. glomerata* L., 'Acaulis' plants were purchased from Campanula Connoisseur (Glenwood Springs, CO). Greenhouse temperatures were maintained at

12–13/10–11 °C, day/night, between December and February and at temperatures ranging between 22/15 °C (March–May), 27/19–21 °C (June–July), 24–29/17–24 °C (August–September), and 21–23/15 °C (October–November) under a natural photoperiod. Plants were grown in 15 cm pots filled with ProMix BX (Stamford, CT) and received a slow release fertilizer (0.8 g of 14N–6.2P–11.7K) at planting and a liquid fertilizer at 200 ppm N from a water soluble fertilizer (15N–7P–14.6K) once a month. Leaf explants were obtained from 1 year old greenhouse grown plants to establish in vitro cultures.

2.2. Explant preparation and establishment for in vitro culture

Young and expanded leaves were harvested from 1 year old greenhouse grown plants during July, September and October, 1997. Leaves were sterilized in a 15% Clorox solution containing 20 drops/l of Tween 20 for 15 min and then rinsed in sterile water. After sterilization of the whole leaves, the proximal and distal ends of the leaf blade were cut off, leaving 2 cm long leaf blades. Leaves of *C. glomerata*, 'Acaulis' were broad so both sides of the leaf blade were cut and 1 cm (length) × 0.5 cm (width) explants were obtained.

Explants were cultured on a Murashige and Skoog (MS) medium with the macro- and micronutrients, vitamins, sucrose and agar (Sigma, M-9274, St. Louis, MO) as described by Murashige and Skoog (1962). The medium was supplemented with one of the following combinations of plant growth regulators: 0, 1.0, 4.0 and 8.0 mg/l (0, 4.4, 17.6 and 35.2 μM) BA and 0, 0.5, 2.5 and 10 mg/l (0, 0.5, 2.5 and 10.0 μM) NAA. The pH of the medium was adjusted to 6.5. Three explants per petri dish ($60 \times 20\,mm^2$) were placed adaxially on the medium and grown at either 14 or 18 °C under continuous light at an irradiance of either 10 (low light) or 75 μmol m^{-2} s^{-1} (high light) from cool white fluorescent lights. There were five petri dishes per treatment. The percentage of explants with shoots was scored after 4 months on regeneration medium. Regenerated shoots were separated and individual plantlets were grown for subsequent regeneration experiments.

2.3. Regeneration of explants obtained from in vitro grown plants grown under different plant growth regulators, temperature and light irradiance condition

Plants established from in vitro culture were cultured on MS basal salts medium without plant growth regulators for 4 months. They were then subcultured on MS basal salts medium supplemented with a factorial combination of 0, 1.0 and 4.0 mg/l (0, 4.4 and 17.6 μM) BA and 0, 0.01 mg/l (0, 0.05 μM) NAA and maintained at either 14 or 18 °C at either low (10 μmol m^{-2} s^{-1}) or high (100 μmol m^{-2} s^{-1}) light irradiance. Four plantlets were grown in each magenta jar, and leaves were obtained from these plants. The regeneration rate was scored after 4 months of culture as described below.

2.4. Regeneration from explants obtained from in vitro grown plants grown for different duration at low temperature (14 °C)

Explants were obtained from the leaf blades from in vitro grown plants under a low light irradiance and at 18 °C for either 0, 1, 2, or 4 months and then transferred to 14 °C for either 4, 3, 2, 1, or 0 months, respectively. After each treatment, explants were placed on a

regeneration medium consisting of MS basal salts medium supplemented with 4.0 mg/l (17.6 μM) BA and 0.1 mg/l (0.5 μM) NAA, pH 6.5. Regeneration data were collected after 4 months.

2.5. Evaluation of plant growth regulators

Leaf explants obtained from plants grown in vitro for 4 months under a low light irradiance and at 18 °C were placed on MS basal salts medium containing one of the 16 combinations of BA 0, 1.0, 4.0 and 8.0 mg/l (0, 4.4, 17.6 and 35.2 μM) and NAA 0, 0.1, 0.5 and 2.0 mg/l (0, 0.5, 2.5 and 10.0 μM). Data were collected monthly for 4 months. In one test, BA was replaced by N^6-(2-isopentenyl)adenosine (2iP) at the same concentrations in mg/l as BA.

2.6. Data collection and statistical analysis

The regeneration rate was evaluated according to the following rating scale: 0, explant dead; 1, only callus developed; 2, one shoot regenerated; 3, two shoots regenerated; 4, three shoots regenerated; 5, four shoots regenerated; 6, more than four shoots regenerated. More than 10–15 shoots were regenerated from a few *C. glomera*, 'Acaulis' explants which could not be counted easily. Therefore, regeneration rate was used to record the data. Data of regeneration rates were transformed using $(x + 0.5)^{1/2}$ where x was the rating scale and subjected to the analysis of variance and regression analysis (MSTAT, 1989). Each treatment was replicated three times and each replication had 45 explants (three explants per petri dish) except in experiments using greenhouse grown leaves (Table 1).

Table 1
Initial screening of *Campanula* cultivar on the regeneration as influenced by plant growth regulators and light intensity[a]

Species/cultivars	Light irradiance[b]	Plant growth regulators (mg/l of BA + NAA)							
		0 + 0		1 + 0.1		4 + 0.5		8 + 2	
		Rating[c]	Dead (%)[d]	Rating	Dead (%)	Rating	Dead (%)	Rating	Dead (%)
C. glomerata	High	0 ± 0	100/0	0 ± 0	100/0	0.4 ± 0.2	80/0	1.3 ± 0.4	53/0
Acaulis	Low	0 ± 0	100/0	0.5 ± 0.3	80/0	1.8 ± 0.3	7/20	2.0 ± 0.4	7/20
C. latiloba	High	0 ± 0	100/0	0.5 ± 0.2	60/0	1.1 ± 0.3	47/0	0.8 ± 0.3	60/0
Hidcote Amethyst	Low	0 ± 0	100/0	1.2 ± 0.3	13/27	0.4 ± 0.2	0/80	1.5 ± 0.2	0/27
C. persicifolia	High	0 ± 0	67/23	1.3 ± 0.3	13/20	1.6 ± 0.2	20/0	1.6 ± 0.2	0/20
White Bells	Low	0.7 ± 0.3	67/0	1.2 ± 0.3	0/40	2.0 ± 0	0/0	1.6 ± 0.2	0/20
C. persicifolia	High	0.0 ± 0	100/0	0.1 ± 0.1	93/0	0.3 ± 0.2	60/27	1.3 ± 0.3	0/33
Telham Beauty	Low	0.1 ± 0	87/7	1.4 ± 0.3	7/27	2.0 ± 0.0	0/0	1.6 ± 0.2	0/20

[a] Leaf explants were obtained from greenhouse grown plants in September. Data represents regeneration status after 4 months in culture.

[b] Photosynthetic photon flux at 10 μmol m^{-2} s^{-1} (L) and 75 μmol m^{-2} s^{-1} (H) at 18 °C.

[c] Mean ± standard error.

[d] Percentage of explant that were dead/dead due to a contamination.

The sampling error (variation among explants) was generally smaller and was not significant as compared to the experimental error and, therefore, the means of three explants per petri dish were subjected to data analysis.

3. Results and discussion

3.1. Regeneration from greenhouse grown explants

Low regeneration and high contamination rate was observed in *C. persicifolia* L. 'White Bells', 'Telham Beauty', and *C. latiloba* A. DC., 'Hidcote Amethyst' (Table 1) when explants were obtained from greenhouse grown plants, *C. glomerata*, 'Acaulis', which showed the highest regeneration rate was, therefore, selected for all subsequent experiments. During the initial test for regeneration, 'Acaulis' explants exposed to high light intensity (75 μmol m^{-2} s^{-1}) failed to produce callus. Explants under low light irradiance (10 μmol m^{-2} s^{-1}) remained green and produced calli after 3 weeks of culture.

The regeneration rate as influenced by light irradiance and plant growth regulators was not statistically different when explants were obtained from greenhouse grown plants in July–September (data not presented). This could be in part due to a low regeneration rate and in part due to a contamination problem (Table 1). However, regeneration was consistently high (average of 41% from regeneration rates of July–September) when explants were cultured on MS basal salts medium supplemented with 4.0 mg/l BA and 0.1 mg/l NAA followed by 36% with 8.0 mg/l BA and 0.5 mg/l NAA. Very few explants of *C. glomerata*, 'Acaulis' when cultured on MS medium supplemented with 8.0 mg/l BA and 0.5 mg/l NAA produced more than 10–15 shoots which was difficult to count. Therefore, a regenerating rating was used in all subsequent experiments. If explants were obtained from young leaves from vernalized plants in the spring, the regeneration rate could have been higher. Explants of *Pelargonium* × *hortorum* obtained from young and expanding leaves regenerated better than those from expanded leaves and old shoots (Agarwal and Ranu, 2000). Culturing the explants of in darkness for a certain period yielded high percentage of shoot regeneration apricot, *P. armeniaca* (Perez-Tornero et al., 2000). Since low irradiance increased the regeneration rate in *Campanula* in this study, keeping the culture in darkness for some period might produce a similar result.

3.2. Regeneration from explants obtained from in vitro plants grown under different light irradiance and temperature conditions

Plants grown in vitro on MS basal salts medium containing 4.0 mg/l BA produced many shoots, but the leaf size was too small and variable to obtain uniform explants of $0.5 \times 0.5\,cm^2$. When in vitro grown plants were subcultured on MS basal salts medium containing 1.0 mg/l BA, the number of shoots formed was intermediate, and their leaves were larger than those grown on 4.0 mg/l BA. The addition of 0.01 mg/l NAA in combination with 1.0 mg/l BA did not affect shoot formation and leaf size, but increased regeneration rates slightly from 3.1 to 3.5 (data not presented). Therefore, plants were

Table 2
Influence of light irradiance and temperature for in vitro grown plants on regeneration rate after 4 months on MS basal salts medium supplemented with 4.0 mg/l BA and 0.1 mg/l NAA[a]

Light irradiance ($\mu mol\ m^{-2}\ s^{-1}$)	Temperature (°C)	Regeneration rate
10	14	3.6
10	18	2.4
100	14	0.8
100	18	0.4
Level of significance		
Light irradiance		**
Temperature		**
Light irradiance × temperature		**

[a] Explants were obtained from in vitro grown plants on MS basal salts medium supplemented with 1.0 mg/l BA and 0.01 mg/l NAA.
** Significant at $P = 0.01$.

cultured in vitro on MS basal salts medium containing 1 mg/l BA and 0.01 mg/l NAA to obtain explants from leaf blades.

The regeneration rate was 4–6 times higher when explants were obtained from in vitro grown plants at 14 °C rather than at 18 °C, and higher when in vitro grown plants were grown under a low light irradiance rather than high light irradiance (Table 2). The average regeneration rate was 3.6, which is equivalent to 2–3 shoots per explant when explants from in vitro grown plants were cultured at 14 °C and 10 $\mu mol\ m^{-2}\ s^{-1}$. Therefore, for all subsequent regeneration experiments, leaf explants were obtained from in vitro grown plants on MS basal salts medium containing 1.0 mg/l BA and 0.01 mg/l NAA and maintained at 14 °C under the low light irradiance conditions.

Table 3
Regeneration of explants obtained from in vitro plants grown for different duration at 14 °C followed by growing at 18 °C before obtaining explants[a]

No. of months at		Regeneration rate
14 °C	18 °C	
0	4	0.79
1	3	1.49
2	2	3.25
3	1	2.60
4	0	2.52
Level of significance		
Duration		
Linear	*	
Quadratic	**	

[a] Data were collected after 4 months on MS basal salts medium supplemented with 4.0 mg/l BA and 0.1 mg/l NAA.
* Significant at $P = 0.05$.
** Significant at $P = 0.01$.

3.3. Regeneration from explants obtained from plants grown in vitro for different duration at low temperature (14 °C)

Explants obtained from plants grown in vitro at 18 °C and low light irradiance showed a low regeneration rate (0.8) (Table 3). Explants obtained from in vitro grown plants at 14 °C for 1 month showed a low regeneration rate (1.5). Explants obtained from plants grown in vitro at 14 °C for 2 months showed a high regeneration rate (3.25). The regeneration rate was influenced by a quadratic effect ($P = 0.01$, $r^2 = 0.79$) of low temperature duration. Culturing plants in vitro longer than 2 months at 14 °C did not significantly improve the

Table 4
Regeneration rate of leaf explants of *C. glomerata* 'Acaulis' as influenced by BA or 2iP (in combination with NAA)[a]

Concentration (mg/l)		BA		Concentration (mg/l)		2iP	
BA	NAA	Rating	Highest rating	2iP	NAA	Rating	Highest rating
0	0	0	0	0	0	0	0
0	0.1	0.5	4	0	0.1	0.8	4
0	0.5	1.0	3	0	0.5	1.4	4
0	2.0	1.0	2	0	2.0	1.3	3
1	0	0.9	5	1	0	1.4	6
1	0.1	3.5	6	1	0.1	2.3	6
1	0.5	2.3	5	1	0.5	2.8	6
1	2.0	1.2	3	1	2.0	2.6	4
4	0	1.8	6	4	0	0.4	3
4	0.1	3.6	6	4	0.1	2.9	5
4	0.5	2.6	4	4	0.5	2.7	5
4	2.0	0.8	3	4	2.0	1.6	3
8	0	1.3	5	8	0	0.8	3
8	0.1	3.5	6	8	0.1	1.9	4
8	0.5	2.8	4	8	0.5	1.3	4
8	2.0	0.7	2	8	2.0	1.0	3
Regression analysis for rating							
Plant growth regulator BA vs. 2iP**							
BA × NAA test				2iP × NAA test			
BA				2iP			
Linear	**			Linear	*		
Quadratic	**			Quadratic	**		
NAA				NAA			
Linear	**			Linear	**		
Quadratic	**			Quadratic	**		
				BA × NAA-linear	*		
				2iP × NAA-linear	*		
r^2	0.47			r^2	0.39		

[a] Data were collected after 4 months. Explants were grown under low light irradiance (10 μmol m^{-2} s^{-1}) at 18 °C.

* Significant at $P = 0.05$.

** Significant at $P = 0.01$.

regeneration rate. The low regeneration rate when explants were obtained from greenhouse grown plants from July to September (data not presented) and in September (Table 1) could partly be attributed to the high light irradiance and high temperatures when plants were grown in the greenhouse.

3.4. Evaluation of plant growth regulators

All explants died when cultured on MS basal salts medium without both cytokinin and auxin (Table 4). The overall regeneration rate was higher when BA was used as a source of cytokinin as compared to 2iP. The average regeneration rate and highest rating of an individual explant was 3.6 and 6, respectively, when explants were cultured on MS basal salts medium supplemented with 4.0 mg/l BA and 0.1 mg/l NAA. With 0.1 mg/l NAA, regeneration rate was not affected by supplemental BA between 1.0 and 8.0 mg/l. With 2iP, shoot regeneration was highest (average regeneration rate of 2.8) when explants were cultured on 1.0 mg/l 2iP and 0.5 mg/l NAA.

When BA was used as compared to 2iP, the shoot regeneration rate from leaf explants was generally higher after 4 months in culture, however, explants regenerated faster with the optimal concentration of 2iP and NAA as compared to the optimal BA and NAA combination (Fig. 1). The regeneration rate became approximately equal by the second month, but the final rate after 4 months culture was higher for 4.0 mg/l BA and 0.1 mg/l NAA than 1.0 mg/l 2iP and 0.5 mg/l NAA. In *Gardenia*, BA produced more regenerated shoots than 2iP (Al-Juboory et al., 1998). The interaction between the source of cytokinin

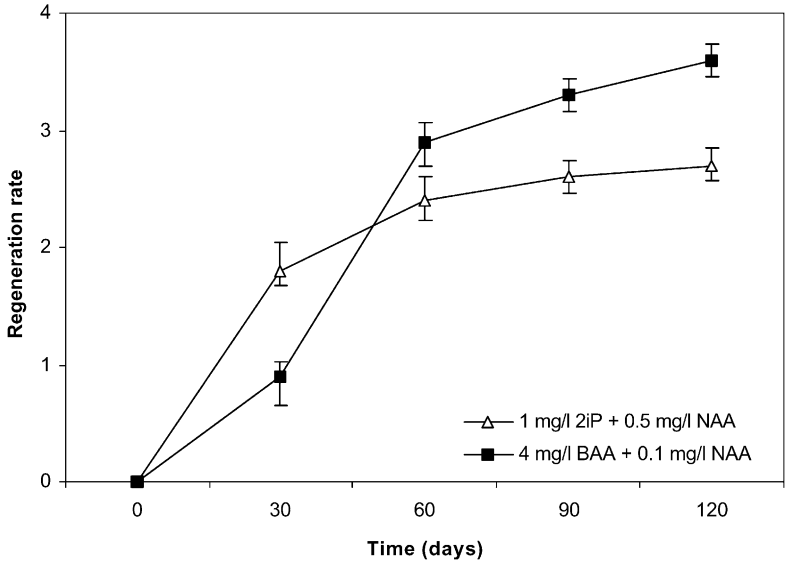

Fig. 1. Regeneration rate of explants of *C. glomerata* 'Acaulis' grown on two plant growth regulator combinations as influenced by time in culture. Bars indicate mean ± standard error (45 explants × 3 replications/treatment).

and the duration of in vitro culture on regeneration may be attributed to the differences in stability of BA and 2iP during culture as BA was reported to get degraded very slowly when compared to 2iP (Brzobohatý et al., 1994). To further increase the regeneration rate, thidiazuron could be used instead of BA as was reported in *P. armeniaca* (Perez-Tornero et al., 2000).

4. Conclusion

The optimal conditions tested for growing plants in vitro to prepare explants from leaf blades were to grow the plants on MS basal salts medium containing 1.0 mg/l BA and 0.01 mg/l NAA, at a low temperature (14 °C) and low light irradiance (10 μmol m^{-2} s^{-1}) for at least 2 months. The optimal conditions for regeneration from leaf explants were using MS basal salts medium containing 4.0 mg/l BA and 0.1 mg/l NAA at 18 °C and a low light irradiance. Regenerated plants after 4 months of culture formed fibrous roots and 11 'Acaulis' plants have been transplanted successfully to soil in a greenhouse. They developed normal morphological characteristics and are being grown in a greenhouse to observe growth and flowering. This is the first report of a successful regeneration of *Campanula* from leaf blade explants and this system has been applied to develop transgenic plants of *Campanula* using *Agrobacterium* (Joung et al., 1999, 2000).

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